LAD-1, the Linear IgA Bullous Dermatosis Autoantigen, Is a Novel 120-kDa Anchoring Filament Protein Synthesized by Epidermal Cells

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This study characterizes a novel basement membrane component that is the target of autoantibodies in patients with linear IgA bullous dermatosis. Tissue surveys showed that this protein localized to the epidermal side of 1 M NaCl split skin and to basement membranes in cornea, oral mucosa, esophagus, intestine, kidney collecting ducts, ureter, bladder, urethra, and thymus, but was absent in lung, blood vessels, skeletal muscle, and nerve. Monoclonal antibody 123, which recognizes this protein, induced dermal-epidermal separation of human skin *in situ*, and this protein was found, by immunoelectron microscopy, to localize exclusively to anchoring filaments. This protein was secreted as a 120-kDa peptide from primary cultures of keratinocytes as determined by radioimmunoprecipitation. Monoclonal antibody 123 recognized this protein as a 120-kDa band from conditioned cell culture medium and a 97-kDa band from human skin extracts as shown by immunoblot. Serum from five patients with the autoimmune blistering disorder linear IgA bullous dermatosis specifically recognized bands of 120 and 97 kDa from culture medium and skin extracts, respectively, that were of identical electrophoretic migration to the bands recognized by monoclonal antibody 123. In summary, this study characterizes a novel anchoring filament protein that is the target of linear IgA bullous dermatosis autoantibodies. Because monoclonal antibody 123 induces blistering of human skin, we hypothesize that this protein functions to maintain dermal-epidermal cohesion and that autoantibodies in this disease are themselves pathogenic. We propose LAD-1 as the name for this protein. *Key words: lamina lucida/basement membrane/autoimmune. J Invest Dermatol 106:734–738, 1996*

Anchoring filaments have been shown to be of critical importance in the maintenance of dermal-epidermal cohesion at the level of the lamina lucida (Burgeson, 1993; Marinkovich, 1993). Two known components of anchoring filaments include laminin-5 (kalinin/nicein/epiligrin) (Rousselle *et al*, 1991; Marinkovich *et al*, 1992a) and laminin-6 (Marinkovich *et al*, 1992b). Laminin-1 is another member of the laminin family of molecules that is present in the lamina lucida of the basement membranes of the epidermis as well as many other tissues (Timpl and Dziadek, 1986).

Linear IgA bullous dermatosis (Chorzelski *et al*, 1979) is an acquired subepidermal blistering disorder characterized by linear deposits of IgA autoantibodies at the dermal-epidermal basement membrane. Chronic bullous disease of childhood is generally regarded as a variant of this disease that affects the pediatric population. Immunoelectron microscopy studies have shown that the IgA autoantibodies localize to the lamina lucida in some patients and to the sub-lamina densa region in others (Bhogal *et al*, 1987). Previously, a group of linear IgA bullous dermatosis patients were shown to have autoantibodies that localized to the lamina lucida and that recognized a 97-kDa peptide in human skin extracts by Western blot analysis (Zone *et al*, 1990). We show in this study that this protein is a novel component of epithelial basement membranes. Further, we demonstrate the structure, tissue distribution, and ultrastructural location of this molecule. Finally, this study also provides information regarding the function and pathologic role of this molecule in linear IgA bullous dermatosis.

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Abbreviation: IgA, immunoglobulin A; MoAb, monoclonal antibody.

MATERIALS AND METHODS

The inoculant for monoclonal anitbody (MoAb) 123 was prepared from the PF1 fraction of pepsin-solubilized human amnion by a previously described procedure (Maddox *et al*, 1989). Murine hybridomas were produced and screened by indirect immunofluorescent microscopy (Sakai *et al*, 1986). Polyclonal rabbit antiserum to laminin-5 (Marinkovich *et al*, 1992a), and anti-laminin-5 MoAb BM165 have been previously characterized (Rousselle *et al*, 1991). Type VII MoAb (clone NP185) was generously donated by Dr. Lynn Sakai of the Shriners Hospital (Portland, OR) and has been previously characterized (Sakai *et al*, 1986). Affinity-purified polyclonal rabbit antiserum to laminin-1, anti-mouse immunoglobulin G, anti-rabbit immunoglobulin G fluorescein isothiocyanate conjugates, anti-rabbit immunoglobulin G, and anti-human IgA horsradish peroxidase conjugates were purchased from Sigma Chemical Co. (St. Louis, MO). Patient serum

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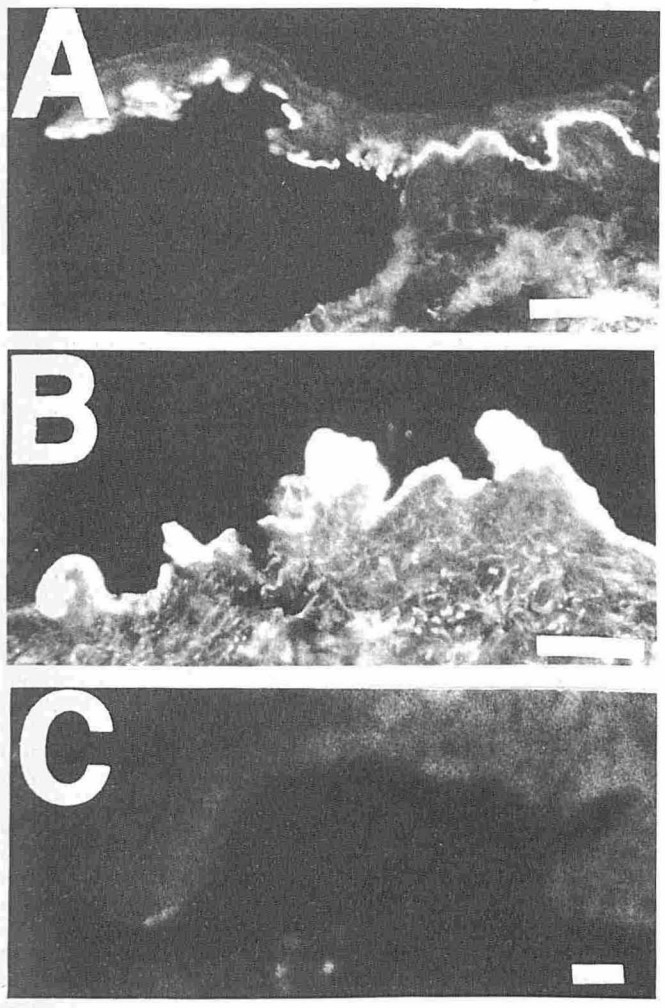

Figure 1. The 123 antigen localizes to the epidermal side of 1 M NaCl split human skin. Frozen sections (8 μm thick) of 1 M NaCl-separated human skin were incubated with MoAb 123 (*A*), MoAb BM165 (*B*), or no primary antibody (*C*), washed, and visualized with anti-mouse IgG-fluorescein isothiocyanate. *Scale bars*, 50 μm.

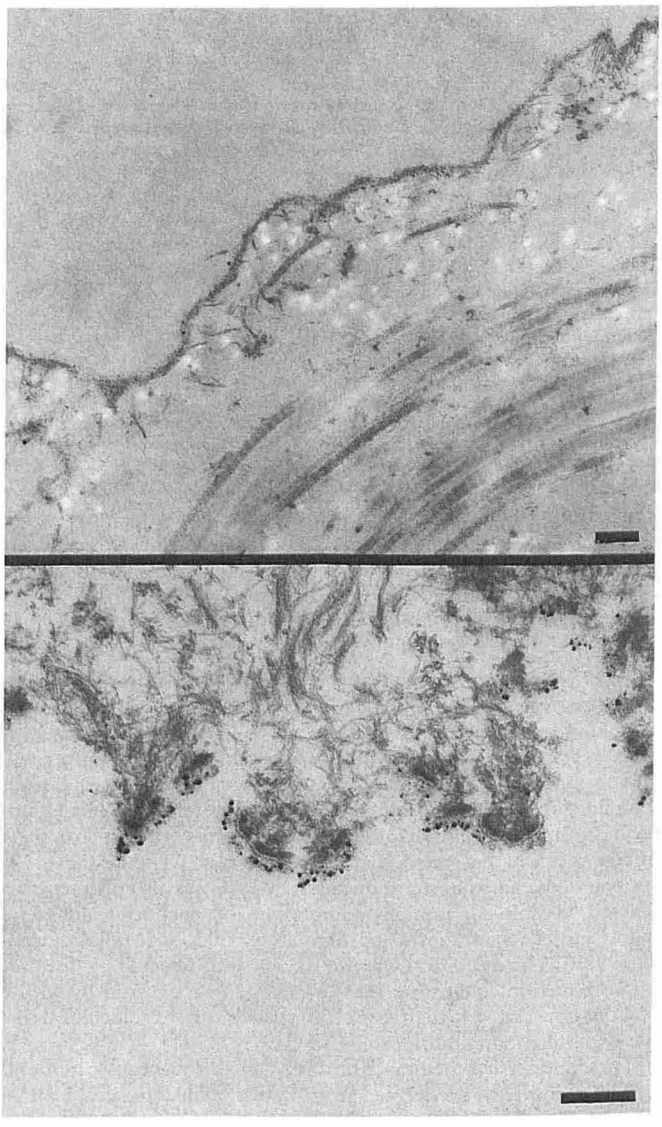

Figure 2. The 123 antigen is an anchoring filament component involved in dermal-epidermal cohesion. Unfixed skin sections were incubated en bloc with 100 μg/ml purified MoAb 123 in PBS, followed by anti-mouse IgG 5-nm immunogold labeling and silver enhancement. *Top*, dermis; *bottom*, epidermis. *Scale bars*, 250 nm.

samples were obtained from previously reported individuals with linear IgA bullous dermatosis (Zone *et al*, 1990).

Conditioned serum-free medium and cell fractions from the SCC-25 cell line (American Type Culture Colection) were prepared as previously described (Marinkovich *et al*, 1992a). 1 M NaCl induced separation of human skin and its extraction (Zone *et al*, 1990), indirect immunofluorescent microscopy (Sakai *et al*, 1986), Western blot analysis of proteins separated by sodium dodecyle sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Lunstum *et al*, 1986) and 2-dimensional SDS-PAGE (O'Farrell, 1975), en bloc immunolabeling of human skin sections followed by silver-enhanced immunoelectron microscopy (Rousselle *et al*, 1991) and immunoprecipitation of [^{35}S]methionine/cysteine-labeled conditioned keratinocyte medium were performed as described (Marinkovich *et al*, 1992a).

RESULTS

Antigen 123 Localizes to Anchoring Filaments In initial immunofluorescent experiments, MoAb 123 was noted to localize to the dermal-epidermal junction of human skin. This MoAb localized to the epidermal side of 1 M NaCl separated human skin, in contrast to anti-laminin-5 MoAb BM165 which localized to the dermal side of the split **(Fig 1)**. The localization of this antibody was then examined by immunoelectron microscopy as shown in **Figure 2**. It was found that, when unfixed human skin was incubated en bloc with a dilute concentration of MoAb 123 (100 μg/ml) in phosphate-buffered saline (PBS) at 4°C for 2 h, followed by washing and secondary antibody incubation and fixation, the epidermis extensively detached from the dermis. The MoAb 123 was noted to localize exclusively to the epidermal rather than the dermal aspect of the induced split. Specifically, the antigen recognized by MoAb 123 localized to anchoring filaments underlying hemidesmosomes.

A tissue survey was performed on fetal bovine tissue to compare the pattern of reactivity of MoAb 123 with that of antibodies against other known basement membrane proteins **(Table I)**. The distribution of MoAb 123 reactivity included the corneal basement membrane as well as the basement membranes that underlie the epithelial linings of many tissues of the gastrointestinal and genitourinary tracts. Unlike laminin-5, the 123 antigen did not localize to alveolar basement membranes. Unlike laminin-1 and other ubiquitous basement membrane components, the 123 antigen did not

Table I. The 123 Antigen Shows a Unique Tissue Distribution*

Tissue	123 Antigen	Laminin-5	Collagen VII	Laminin-1
Skin	++	++	++	++
Cornea	++	++	++	++
Lung	−	++	±	++
Esophagus	++	++	++	++
Small intestine	+	±	++	±
Ureter	++	++	++	++
Bladder	++	++	++	++
Thymus	++	++	++	++
Peripheral Nerve	−	−	−	++
Skeletal muscle	−	−	−	++
Blood vessels	−	−	−	++

* A 24-inch crown to rump length fetal bovine calf was used for all tissues except amnion which was human and obtained from a normal term delivery. Indirect immunofluorescent microscopy was performed on frozen sections of all tissues using antibodies against the indicated proteins as described in *Materials and Methods*. ++, strong staining; +, weak staining; ±, negligible staining; −, absent staining.

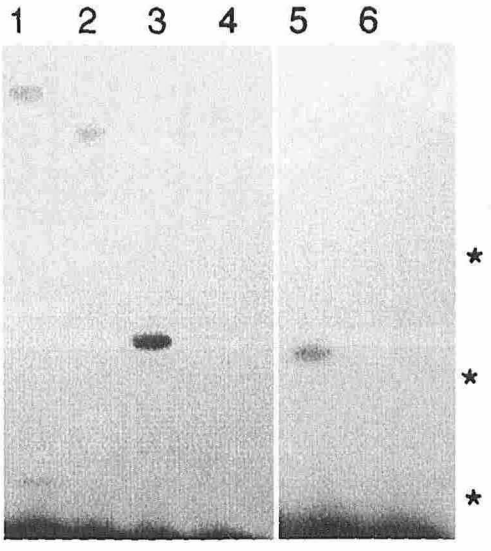

Figure 3. The 123 antigen is synthesized by human keratinocytes. Human neonatal keratinocytes were radiolabeled with 100 μCi of [^{35}S]cysteine/methionine in cysteine/methionine-deficient medium for 24 h. Labeled medium was immunoprecipitated, separated by nonreduced (*lanes 1–4*) or reduced (*lanes 5* and *6*) SDS-PAGE, and visualized by fluorography using the following primary antibodies: *lane 1*, anti-laminin-1 antiserum; *lane 2*, laminin-5 antiserum; *lanes 3* and *5*, MoAb 123; *lanes 4* and *6*, no primary antibody. Positions of 205-, 116-, and 77-kDa molecular weight markers are indicated on the right.

localize to neural, endothelial, or skeletal muscle basement membranes. Unlike the 123 antigen, integrin α6 subunit localized to dermal endothelial basement membranes (not shown).

The 123-Antigen Is Distinct from Other Known Proteins The structure of the antigen recognized by MoAb 123 was examined by immunoprecipitation of ^{35}S-labeled conditioned keratinocyte medium. A single 120-kDa peptide was immunoprecipitated by MoAb 123. This peptide was distinct in its electrophoretic mobility on nonreduced SDS-PAGE relative to two other lamina lucida components laminin-1 and laminin-5 **(Fig 3)**. Based on these findings, it can be stated that the molecular weight of the 123 antigen deduced from nonreduced SDS-PAGE is also distinct from that of laminin-6, another component of anchoring filaments whose molecular weight is approximately 650 kDa (Marinkovich *et al*, 1992b). The migration was not significantly affected by disulfide bond reduction, indicating that the antigen recognized by MoAb 123 did not form covalent associations with other molecules under the experimental conditions employed.

The conditioned medium from SCC-25, a squamous carcinoma cell line, and extracts derived from human skin separated by 1 M NaCl both proved to be good biological sources of 123 antigen. Although a faint band at 120 kDa was detected in the cell fraction of SCC-25 cells, the majority of the antigen recognized by MoAb 123 was present in the culture medium of these cells **(Fig 4)**. These results suggest that the 123 antigen is a secreted protein, unlike BP180 or integrin α6β4, which are transmembrane, cell-associated proteins.

In contrast to the 120-kDa band seen in culture-derived material, MoAb 123 recognized a 97-kDa band in human skin extracts. The pattern of bands recognized by polyclonal antibodies against laminin-1 and laminin-5 are clearly of different electrophoretic mobility compared to the bands recognized by MoAb 123. In the SCC-25 medium sample, the 120-kDa band recognized by MoAb 123 migrated to a position about midway between the 140-kDa β3 subunit of laminin-5 and the 105-kDa processed laminin-5 γ2 subunit. These studies also indicate that the electrophoretic mobility of this antigen is distinct from that of BP180 or the β4 integrin subunit.

The 123 Antigen Is Recognized by Linear IgA Bullous Dermatosis Autoantibodies Comparative Western blot studies were performed to determine whether the antigen recognized by MoAb 123 was related to a 97-kDa peptide in skin previously reported to be recognized by linear IgA disease patient autoantibodies (Zone *et al*, 1990). As seen in **Fig 5**, IgA autoantibodies in serum samples from five patients with linear IgA disease recognized 120- and 97-kDa bands from SCC-25-conditioned medium and human skin extracts, respectively, that were of identical electrophoretic mobility compared to the bands recognized by MoAb 123. This mobility was also identical on 2-dimensional gel electrophoresis. As shown in **Fig 6**, MoAb 123 as well as another linear IgA bullous dermatosis sera sample both recognized a slightly acidic 97-kDa band (a minor band of slightly higher molecular weight also recognized by this patient's sera is of uncertain identity). These results indicate that the 123 antigen is the target of linear IgA bullous dermatosis autoantibodies in these patients. Based on these findings, we propose the name LAD-1 (linear IgA bullous dermatosis antigen 1) for this protein.

We have very recently noted that MoAb 123 and IgA autoantibodies consistently detect a 120-kDa band in skin extracts that comigrates with the band derived from cell culture when more extensive protease inhibition is utilized in the skin extraction process (not shown), suggesting that this protein exists as 120 kDa *in vivo* and that the 97-kDa peptide is a degraded rather than specifically processed product.

DISCUSSION

In this study, we have used several methods to demonstrate that LAD-1, the antigen recognized by MoAb 123, has features that are distinct from other known basement membrane proteins. For example, it appears to be the only completely extracellular protein that localizes to the epidermal side of NaCl split basement membrane. In this regard, it appears to be distinct from a previously described 105-kDa protein (Chan *et al*, 1995) in that this protein localizes to the dermal aspect of NaCl split skin. This evidence, combined with the immunoprecipitation and Western blot data presented in this study suggest that LAD-1 is distinct from laminin-5, laminin-6, and laminin-1. LAD-1 appears distinct from α6β4 integrin in that it predominantly localizes to the medium rather than to the cell fraction of cultured epidermal cells. The molecular weight of LAD-1 differs from that of the β4 integrin subunit (200 kDa) and the tissue distribution of the α6 integrin subunit is distinct in that it localizes to blood vessels while LAD-1 does not.

It was noted that MoAb 123 or linear IgA patient sera do not

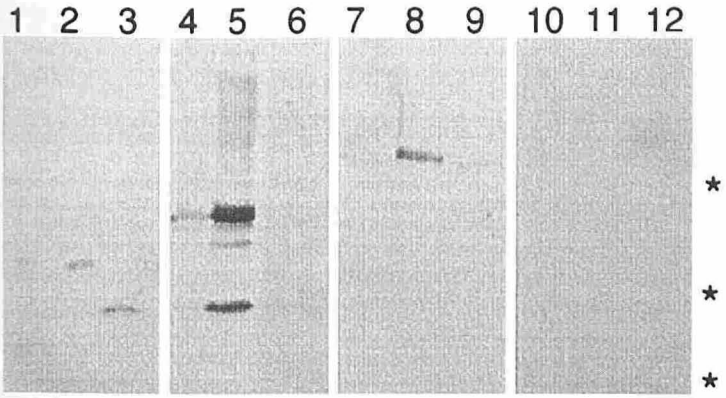

Figure 4. 123 antigen is present in human skin extracts and conditioned cell medium, and is distinct from other lamina lucida proteins. Samples of SCC-25 cells (*lanes 1, 4, 7, 10*), SCC-25-conditioned medium (*lanes 2, 5, 8, 11*), and human skin extract (*lanes 3, 6, 9, 12*) were analyzed by Western blot using the following primary antibodies: MoAb 123 (*lanes 1–3*); anti-laminin-5 antiserum (*lanes 4–6*); anti-laminin-1 antiserum (*lanes 7–9*); no primary antibody (*lanes 10–12*). Positions of the bands recognized by MoAb 123 are indicated on the left. Positions of 205-, 116-, and 77-kDa molecular weight markers are indicated on the right.

recognize a 180-kDa BP180 band in immunoblots of SCC-25 extracts or human skin **(Fig 4)**, even though a 180-kDa band is recognizable in these samples in immunoblot using BP180 antibodies (not shown). This evidence coupled with the observed difference in apparent molecular weight between LAD-1 and BP180 and the observation that LAD-1 appears to be a secreted protein suggest that this antigen is not BP180.

A 125-kDa lamina lucida constituent of bovine tongue mucosa has been previously described (Klatte *et al*, 1989) and has been shown to differ in its pattern of expression compared to the 240- and 180-kDa bovine bullous pemphigoid antigens. The MoAb that recognizes this peptide does not recognize human tissue; however, MoAb 123 recognizes both human and bovine tissue immunoblot and was found to specifically recognize the 125-kDa bovine peptide (J.C.R. Jones, personal communication). Thus, LAD-1 appears to be the human counterpart to a previously identified bovine basement membrane antigen.

LAD-1 is not likely to be the antigen recognized by MoAb 19-DEJ-1 (Fine *et al*, 1989) recently termed uncein. Uncein has been preliminarily shown to consist of three subunits of 165 kDa, 130 kDa, and 100 kDa.[1] Unlike uncein, LAD-1 consists of a single peptide of 120 kDa. Furthermore, the molecular weight of LAD-1 is not altered by disulfide bond reduction, implying that, unlike uncein, it does not form multiple subunit associations.

It is well known that 1 M NaCl induces dermal-epidermal separation of human skin in the mid-lamina lucida region (Gammon *et al*, 1992). This phenomenon suggests that noncovalent interactions are involved in the maintenance of dermal-epidermal cohesion at this level. NaCl-induced separation can be used to deduce the location of components within the lamina lucida. Both laminin-5 and LAD-1 localize to areas directly underlying hemidesmosomes in the region of anchoring filaments; however, because LAD-1 localized to the epidermal aspect of the split, it appears to be located higher in the lamina lucida and closer to the hemidesmosome than laminin-5, which localized to the dermal aspect of 1 M NaCl separated skin.

Despite our extensive experience with en bloc incubation of human skin with antibodies against a variety of basement membrane components, only two antibodies, MoAb 123 (this report) and anti-laminin-5 MoAb BM165 (Rousselle *et al*, 1991) have been shown to induce dermal-epidermal separation. Despite their common ability to induce epidermal separation, MoAb 123 and MoAb BM165 appear to induce this separation by different mechanisms. Previously MoAb BM165 was shown to localize to the dermal aspect of the antibody-induced split (Rousselle *et al*, 1991). In contrast, MoAb 123, as shown in this study, localized to the epidermal aspect of the induced split.

These *in situ* experiments correlate with *in vivo* observations of two groups of patients with intra-lamina lucida skin blistering diseases. One subset of cicatritial pemphigoid patients have been shown to have circulating autoantibodies against laminin-5 (Kirtschig *et al*, 1995). In these patients, the autoantibodies localize exclusively to the dermal floor of the blister. In contrast, the lamina lucida autoantibodies in patients with linear IgA bullous dermatosis localize to the epidermal blister roof (Zone *et al*, 1990). A likely explanation for both the *in situ* and *in vivo* blistering observed with antibodies to LAD-1 and laminin-5 is that the antigen function is disrupted by antibody-induced displacement of a cohesive ligand from its binding site on the antigen. This explanation would predict that the lamina lucida autoantibodies in patients with linear IgA

[1] Zeng L, Riddelle K, Daniels A, Bloom K, Fine JD: Molecular cloning of uncein, an anchoring filament protein abnormally expressed in all junctional epidermolysis bullosa (JEB) skin-partial sequencing of a completely novel gene. *J Invest Dermatol* 104:582, 1995 (abstr.).

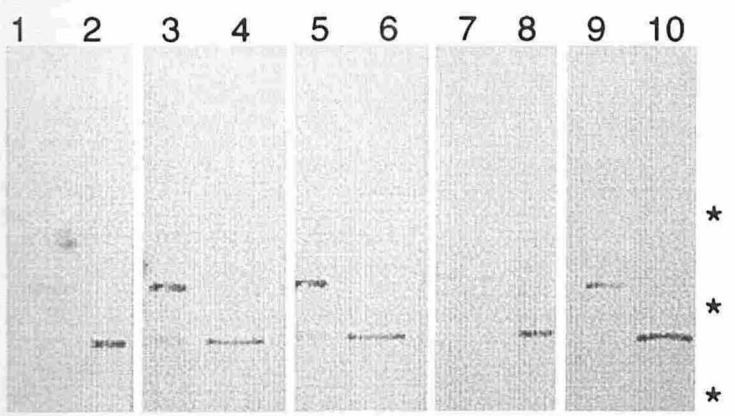

Figure 5. 123 antigen is recognized by linear IgA bullous dermatosis autoantibodies. SCC-25-conditioned medium (*odd-numbered lanes*) or human skin extracts (*even-numbered lanes*) were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose, incubated with serum from five patients with linear IgA bullous dermatosis (patient 1, *lanes 1* and *2*; patient 2, *lanes 3* and *4*; patient 3, *lanes 5* and *6*; patient 4, *lanes 7* and *8*; patient 5, *lanes 9* and *10*) at dilutions between 1:40 to 1:100 and visualized with horseradish peroxidase-IgA conjugate. Positions of 205-, 116-, and 77-kDa molecular weight markers are indicated on the right.

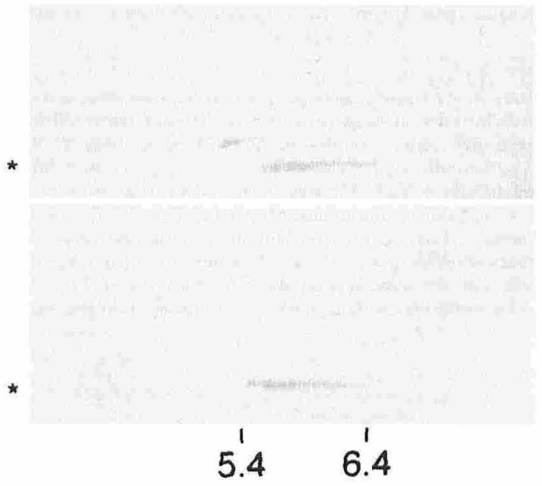

Figure 6. 123 antigen/linear IgA bullous dermatosis autoantigen is an acidic protein. Human skin extract was separated by 2-dimensional SDS-PAGE and analyzed by Western blot using linear IgA patient autoantibody (*top*) or MoAb 123 (*bottom*). pH positions are shown below.

disease are themselves pathogenic. Further animal studies are needed to confirm this hypothesis.

Linear IgA bullous dermatosis patient autoantibodies that localize to the sub-lamina densa region have been reported (Bhogal *et al*, 1987), although we have not observed this pattern of IgA deposition in our patients. Clearly some other pathophysiologic mechanism must be proposed in these cases.

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